IS OSMOTIC HEMOLYSIS AN ALL-OR-NONE PHENOMENON?

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I

There are at present two opposed views concerning the relation between the disappearance of the erythrocyte and the amount of hemoglobin which it liberates during osmotic hemolysis. Some investigators (Ruzynyák, S., 1911; von Lieberman and von Fenyvessy, 1912; and J. Baron, 1928) believe that the disappearance is a gradual process resulting from a slow escape of hemoglobin, and hence that swollen though visible cells may have lost considerable quantities of this substance. Baron, for example, has reported that hemolysis induced by hypotonic salt solution may lead, as in one experiment which he cites, to the disappearance of 17 per cent of the cells while at the same time the amount of hemoglobin recovered in the surrounding solution is as high as 42 per cent. The conclusion is drawn that even visible cells must have lost a part of their hemoglobin. Baron and others have designated hemolytic processes of this type as "partial."

Another group of investigators (Dienes, L., 1911; H. Handovsky, 1912; S. C. Brooks, 1918; and G. Saslow, 1928–29) hold that the erythrocyte disappears with great rapidity at the time of hemolysis. Brooks, who worked chiefly with hemolysis by ultra-violet radiation, has summarized this concept in the following way: "When hemoglobin finally begins to diffuse from a given erythrocyte, the process is so quickly completed that it may ordinarily be regarded as instantaneous." According to this view the process is of the type customarily termed "all-or-none." A parallel situation is believed by many workers to be found in osmotic hemolysis. It is held by such workers that the cell subjected to osmotic changes which result in swelling, does not begin to lose its hemoglobin until it has attained a fairly definite volume, termed the "hemolytic volume," at which time a rapid outward diffusion of hemoglobin occurs to a point of equilibrium with the surrounding fluid.

The apparent "hemolytic volume" is believed to differ for different erythrocytes, even in blood from the same individual, hence the well-known range of resistance always encountered in experiments on osmotic hemolysis. In general, according to the second view that hemolysis is an all-or-none process, it ought to be possible to find a solution of such a concentration as to cause, in a given sample of blood, a complete loss of hemoglobin from all the cells whose resistance falls below a certain value, without any loss from the cells of higher resistance. According to the first view, however, that partial hemolysis is a phenomenon of common occurrence, such a sharp separation should not be possible.

The difference between these two views is of more than theoretical interest. If the first one is correct then little significance can be attached to the term " percentage hemolysis," and standards such as those used, for example, by Jacobs (1930), while reproducible and therefore of practical value, correspond to nothing encountered in actual experiments. If, on the other hand, hemolysis is actually an all-or-none phenomenon, then such standards represent not merely the apparent but also the true percentage of hemolysis, with a consequent gain in the significance of the results obtained.

The study of the relation between the disappearance of the cell and the liberation of hemoglobin involves measurements of two sorts: first, a count of the number of cells in the sample of blood employed, and second, a determination of the total hemoglobin content of the cells followed by an estimation after hemolysis has occurred of the number of cells undestroyed and of the hemoglobin content either of these cells or of the supernatant fluid, or preferably of both. An obvious point, which has been neglected by previous workers in securing ideal conditions for the outward diffusion of hemoglobin, is the use of a large volume of surrounding solution relative to the total volume of the cells, so that a true diffusion equilibrium would permit the escape of almost all of the hemoglobin that is free to leave the affected cells.

Under conditions where the outward passage of hemoglobin is not limited by an insufficient external volume, a comparison of the number of cells destroyed and the amount of hemoglobin liberated might conceivably lead to any one of three following results: (1) The amount of hemoglobin in the surrounding solution corresponds exactly to that contained in the cells that have disappeared. The process is therefore " all-or-none " in character. (2) The amount of hemoglobin in the surrounding solution is greater than that contained in the cells that have disappeared. This would suggest a " partial " process, at least in the case of some of the cells. (3) The amount of hemoglobin in the surrounding fluid is less than that contained in the cells that have disappeared. Such a condition would indicate a slight retention of hemoglobin by invisible cells. All of these possibilities are illustrated in Table I by data selected from the previous literature or obtained in the course of this investigation.

Strictly speaking, the "retention" type must always be present to some extent when hemolysis occurs in finite volumes of solution since a diffusion process can do no more than bring about an equality of distribution of hemoglobin between the cells and their surroundings. In previous work, where the whole blood was introduced into the hemolytic solution in the volume ratios of 1 to 1 to 1 to 5 (Baron, J., 1928; G. Saslow, 1928–29) this retention must of necessity have been great. In the present work, however, this effect has been minimized by employing a ratio of 1 to 2,000, thus providing an opportunity for an almost complete outward diffusion of the hemoglobin.

TABLE I

Summary of the Possible Types of Osmotic Hemolysis

Type	Cells Disappeared	Hemoglobin Appeared	Hemolysis in	Observer	Maximum Error of Method
	per cent	*per cent*			*per cent*
(1)	50	51	Dilute plasma	Saslow	7
"all-or-none"	75	76	NaCl, glycerol, ethylene glycol	author	4
(2) "partial"	17	42	Dilute plasma	Baron	?
(3) retention	66	58	Glycerol (early stages)	author	4

It should be noted further that a process of type (3) might be expected to occur as a temporary stage in the attainment of end results of type (1). Whether or not it would be observed would depend on the rapidity of the process and upon the conditions governing the visibility of the cells. Theoretically also a combination of types (2) and (3) might conceivably at times simulate type (1). That such a combination of effects would occur so exactly and consistently in an extended series of experiments as to lead to erroneous conclusions is, however, extremely unlikely.

In the present investigation it has been found that the condition described as type (1) is always present when the hemolytic system has attained a final equilibrium. In certain cases, for example, during the course of hemolysis produced by glycerol solutions, a temporary retention of some hemoglobin is exhibited during the early stages of the process; but at final equilibrium the relation is the same as before, namely, "all-or-none." No evidence whatever has been found under the conditions of these experiments of the escape of hemoglobin from visible cells; that is, of "partial hemolysis" of individual erythrocytes.

II

To determine accurately the relation between the number of erythrocytes that disappear and the amount of hemoglobin liberated in a given hemolytic system it is necessary that a great number of careful cell counts be made. In the present work these counts were made with the usual counting chamber, the areas customarily used for white cells being employed, and at least 10 of these areas of 1.0 sq. mm. each being counted. This procedure involved a count of from 1,500 to 2,000 cells for each solution. By counting a large number of unit areas (160) and also a large number of cells the accuracy of the cell count was increased so that the maximum error was 2 per cent as determined by the method of Student (1907). The use of dilute cell suspensions (1 to 2,000) eliminated the difficulties encountered by previous workers owing to the presence of so-called "ghost" cells. This point will be considered in detail later, and has been touched upon here solely to stress the fact that at no time during the course of these experiments did the difficulty which previous investigators (Baron; Saslow) found in distinguishing between "stromata" and intact cells arise.

The method of hemoglobin estimation employed in all of these experiments was a new one particularly well adapted to this purpose. It was worked out in conjunction with Dr. W. R. Amberson and Dr. D. R. Stewart, and will be described in detail elsewhere (1931). The principle involved in the method is that of the optical pyrometer, and its most valuable features are the high sensitivity and accuracy that can be obtained. The sensitivity proved to be entirely adequate to deal with the minute amounts of hemoglobin that the use of very dilute cell suspensions necessitated. Thus, in experiments in which whole blood was mixed with distilled water in the proportion of 1 to 2,000 it was found that between this concentration and zero concentration of hemoglobin a series of at least one hundred readings could be obtained by the pyrometer. This represents a maximum error in the hemoglobin readings even at these unusually low concentrations of 1 per cent. The term "percentage of hemoglobin" is everywhere used in this paper to designate the amount of hemoglobin in the surrounding fluid of a given hemolytic solution relative to that contained in the total number of cells employed.

It is known that whole blood on standing even for a short period of time (2 to 3 hours) and at a low temperature (3° to 5° C.) often undergoes a slight degree of hemolysis which may range between 0 and 4 per cent. The amount of hemoglobin in the plasma introduced with the whole blood into the hemolytic solutions, as well as other pigments that may be present, must, therefore, be corrected for in the hemoglobin determinations. This has been accomplished by diluting whole blood in the proportions of 1 to 2,000 in isotonic saline and then rapidly (10 minutes) centrifuging the cells out. A pyrometer reading on the supernatant fluid gives the error due to the hemoglobin and other pigments in the plasma. This correction for plasma error has been applied in all the experiments here recorded.

The dilution steps involved in the hemoglobin determination, the preparation of the cell suspensions and the cell counts were found to have an error of not more than 1 per cent. Since the cell counts involved an error of approximately 2 per cent and the hemoglobin determinations of not more than 1 per cent, the total maximum error in comparison of the disappearance of cells and of the appearance of hemoglobin in the solution was of the order of magnitude of 4 per cent. It will be noted that in Table II and in Figs. 1 and 2 the agreement is well within this figure.

TABLE II

Data Illustrative of the Accuracy of the Method

A. Percentage of hemoglobin appeared	3	3	12	20	25	30	51	62	77	95
B. Percentage of hemoglobin in cells remaining	99	96	89	78	78	70	50	39	21	4
A + B	102	99	101	98	103	100	101	101	98	99

Table II also gives another very important check on the accuracy of the method in demonstrating that the hemoglobin which was recoverable from the visible cells plus the hemoglobin in the supernatant fluid is equivalent, within the experimental error, to that contained in the original number of cells. There is no evidence that this very necessary test has been made by previous workers and it is apparently to its omission that much of the confusion in the literature is attributable.

A point of particular importance in obtaining the results here recorded is strict attention to the influence of the factors discussed by Jacobs and Parpart (1931), of which temperature, pH and the attainment of a final equilibrium are the most important. A recent worker (Saslow, G., 1928–29) states that "Most of the experiments performed were failures because of the difficulties above enumerated: lack of control of degree of hemolysis and unsatisfactoriness of the cell count."

Only in three instances was he able to secure a suitable degree of hemolysis, and this unfortunately fell within the narrow range of from 40 to 60 per cent. In the present work, by a careful control of the factors influencing the degree of hemolysis, namely temperature, pH and the attainment of equilibrium, and by the use of dilute cell suspensions, thus allowing a practically complete outward diffusion of hemoglobin from the affected cells which removes the necessity for determining "ghosts," it has been found possible to obtain very readily any desired degree of hemolysis. This may be observed in Fig. 1, where the entire range from zero to 100 per cent has been covered with gaps of no more than 5 per cent.

III

Osmotic hemolysis has been used throughout this work. The experiments performed fall into two classes. Those belonging to the first class involved the use of a non-penetrating substance, sodium chloride, in hypotonic solutions of concentrations so chosen as to bring about a disappearance of some but not all of the erythrocytes present in the suspension. By varying the concentration in small steps it was possible to cover the entire range from zero to 100 per cent hemolysis. After the hemolytic system had reached its final equilibrium condition or after hemolysis had been checked in the manner described below, a comparison was made between the number of cells that had disappeared and the amount of hemoglobin that had been liberated. In the second group of experiments hemolysis was allowed to occur in solutions, originally isosmotic with blood, of the penetrating substance glycerol and, in a few cases, ethylene glycol. The hemolysis produced by the penetrating substances was checked at various points by the addition of sodium chloride in the proper amount and comparisons were made as before between the liberation of hemoglobin and the disappearance of cells. Because of their greater simplicity, the experiments involving solely the entrance of water into the cells will be described first, and those involving the penetration of the solute as well will be dealt with in the following section.

As has already been mentioned, the factors of temperature and pH must be so regulated that their effect on the degree of hemolysis attained is a constant one. The temperature of the hemolytic solutions employed in these experiments was maintained at 20° ± 0.1° C. by means of a water bath. The pH of the hypotonic solutions was controlled by the addition of a small amount of phosphate buffer. All solutions were prepared from a stock solution consisting of 14 parts of molar NaCl and 1 part of molar Na_2HPO_4, brought to a pH of 7.0 by the addition of a trace of concentrated HCl. Upon dilution of this stock solution to concentrations between 0.5M and 0.05M, the pH of the resulting solutions was 7.40 within the limits of accuracy of the quinhydrone electrode (± 0.02). Since the pH of the blood used is originally not far from this point, the comparatively slight buffering of the solution is sufficient for all practical purposes.

In the experiments involving the simplest type of osmotic hemolysis the procedure was as follows: To 50 cc. amounts of various hypotonic salt solutions, usually differing from one another by 0.001M, 25 cu. mm. of whole blood, defibrinated by whipping, was added from a calibrated hemoglobin pipette after the solutions had been brought to a temperature of 20° C. ± 0.1°, in a water bath. Following the addition of blood they were gently and continuously stirred, at the temperature stated, for a period of one hour, which is more than sufficient for the attainment of equilibrium (Jacobs and Parpart, 1931). A small portion of the solution was then removed for the cell count, while the rest was centrifuged at 2,000 r.p.m. for 15 minutes and the supernatant fluid siphoned off for the hemoglobin estimation. The time allowed for centrifuging was shown to be sufficient by two procedures, namely, by microscopic examination of the supernatant fluid which revealed the presence of no cells, and by measurement with the optical pyrometer which gave the same transmission values for the fluid whether it had been centrifuged for 10 minutes or for one hour.

TABLE III

Data on the Blood of One Animal (Ox) Illustrating the Applicability of the All-or-none Concept at Equilibrium

Concentration of $NaCl + Na_2HPO_4$	A. Cells Disappeared	B. Hemoglobin Appeared *	B–A
M/l	*per cent*	*per cent*	
0.154	0	0	—
0.115	3	3	0
0.110	9	12	+3
0.100	22	23	+1
0.095	27	30	+3
0.090	64	62	−2
0.085	79	77	−2
0.080	92	95	+3

* After correction for the plasma error.

Table III presents the results obtained in a typical experiment of this sort with ox blood. The concentrations indicated represent dilutions of the original stock solution containing both NaCl and Na_2HPO_4 whose concentration was somewhat arbitrarily taken as unity. Though osmotically the concentrations given are not exactly equivalent to similar ones of pure NaCl, the differences are very small and the solutions are entirely reproducible. The values given in column A represent the difference between the total number of cells employed and the number of cells remaining when the hemolytic system was at equilibrium, and are expressed on a percentage basis. Column B gives the percentage of hemoglobin in the surrounding solution, which was determined after correction for plasma error as previously described.

Inspection of Table III shows that the hemoglobin which appears in the supernatant solution corresponds, within the limits of experimental error, almost exactly with that originally contained in the cells that have disappeared. There is no evidence, therefore, at the end of the time in question (1 hour) of any appreciable retention of hemoglobin nor of partial hemolysis of cells still visible. The process under these conditions appears to be strictly "all-or-none" in character.

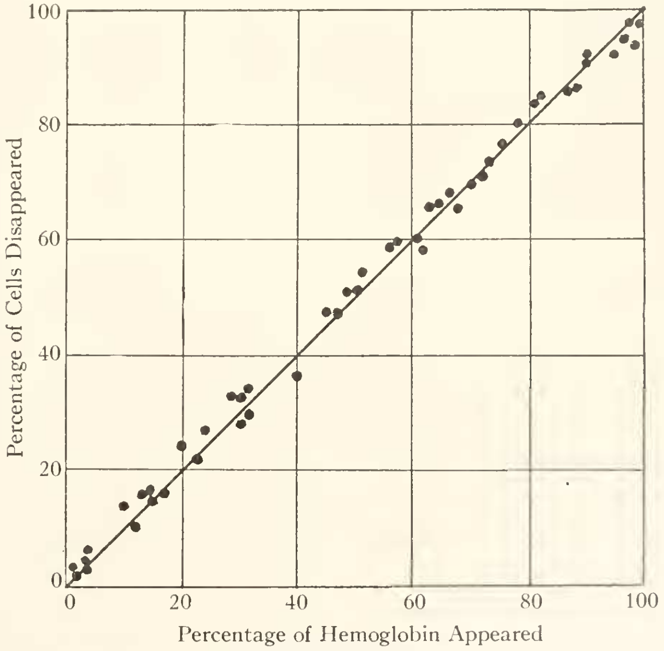

FIG. 1. The liberation of hemoglobin from ox erythrocytes in hypotonic NaCl solutions under equilibrium conditions.

More extensive corroborative data are shown in Fig. 1, where the results of 50 experiments in which the blood of 12 animals was used are plotted. The solid diagonal line represents the condition that should obtain if the hemolytic process, at equilibrium, is all-or-none in character. The experimental points scatter about this line within the error of the method; that is, with a maximum deviation of 4 per cent and an average deviation of 1.7 per cent. Since it is inconceivable that in such a large number of experiments as exact an agreement as this could be obtained by a fortuitous combination of retention of hemoglobin and of partial hemolysis, the conclusion seems inescapable that an all-or-none process is being dealt with.

The results and conclusions summarized in Tables II and III and in Fig. 1 were obtained with ox blood, while the conflicting data of Baron (1928) and Saslow (1928–29) were based upon experiments on human blood. Since there is a marked difference in the osmotic resistance of human blood and of ox blood it seemed advisable to perform a number of similar experiments on the former type of blood. These

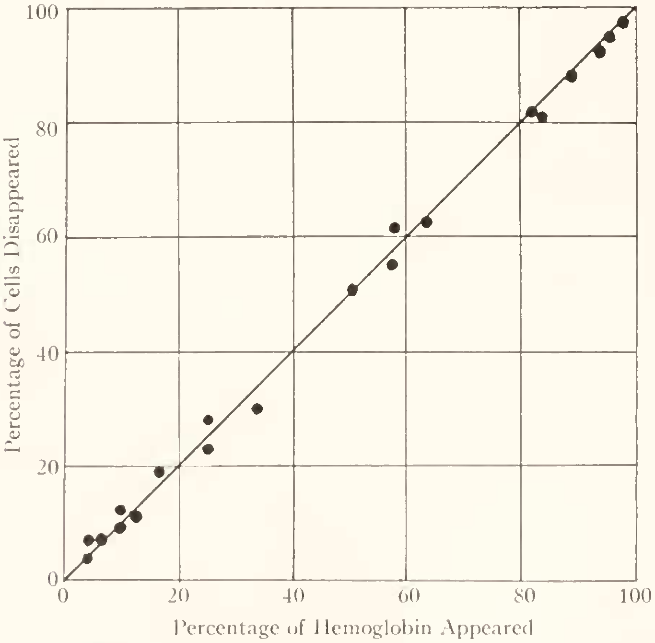

FIG. 2. The liberation of hemoglobin from human erythrocytes in hypotonic NaCl under equilibrium conditions.

experiments are represented in Fig. 2, in which the diagonal line has the same significance as before. It will be noted from this figure that the evidence of an all-or-none relationship is as unmistakable in the case of human as in that of ox blood.

In all of the experiments so far described hemolysis has been allowed to go to complete equilibrium. The question arises whether there is a similar agreement between the hemoglobin that has escaped and the number of cells that have disappeared at times before the final equilibrium of the system has been attained. Stated in a more concrete manner, if in a given hemolytic system the concentration is such that the final equilibrium will involve the destruction of, for example, 75 per cent of the cells present, will the all-or-none concept hold when only 25 per cent of the cells have undergone hemolysis?

To answer this question, the rate of hemolysis of ox blood in hypotonic salt solutions in which at equilibrium there remained some percentage of the cells unhemolyzed, was first followed by the method of Jacobs (1930), and in this way it was determined at what time after setting up a given experiment any desired degree of apparent hemolysis had been attained. It was then possible to repeat the experiment and to stop the hemolysis at the chosen point and to make comparisons as before. In a number of experiments of this type 25 cu. mm. of whole blood of the ox was added to 25 cc. amounts of hypotonic saline solutions whose hemolysis-time curves had been determined as above. At the end of a definite time (15 seconds to 2 minutes) 25 cc. of salt solution of a concentration to make the whole isotonic (0.154M) was suddenly mixed with the hemolyzing solution, thus stopping hemolysis. As before, the temperature was kept at 20° C. and the pH at 7.4. Cell counts were then made on a portion of the solution in which hemolysis had been stopped, while the remainder was centrifuged and the supernatant fluid used for the hemoglobin estimations in the usual manner. A series of these determinations led to the results recorded in Table IV.

It is evident from this table that the agreement between the percentage of the cells that have disappeared and the hemoglobin that has been liberated is as good as it was in the cases where the final equilibrium had been reached. The all-or-none concept, therefore, is not limited merely to the end-stage of osmotic hemolysis of this type but probably applies throughout the entire process. The conclusion would seem to be warranted that in studies on the kinetics of osmotic hemolysis the cell may be assumed to liberate all of its hemoglobin at the time of its disappearance.

IV

Osmotic hemolysis in solutions of penetrating substances is a somewhat more complicated process than that so far described since the rate of entrance into the cell of the solute as well as that of water is involved. Since previous workers have apparently not studied the nature of the hemolytic processes induced by these substances, it appeared of interest to determine whether they might also be associated with an all-or-none type of hemolysis. The substance chiefly studied, namely glycerol, was chosen not only because it is relatively non-toxic, but because its rate of penetration into the erythrocyte of the ox is sufficiently slow so that the hemolytic process can be stopped at any desired point by the addition of salt in proper concentration. A typical experiment may now be described.

TABLE IV

Effect of Stopping Hemolysis Before the Attainment of Equilibrium

Concentration of NaCl + Na_2HPO_4	A. Cells Disappeared	B. Hemoglobin Appeared	B−A
M/l	*per cent*	*per cent*	
0.090	71	72	+1
0.090	31	27	−4
0.090	36	35	−1
0.090	37	38	+1
0.088	75	74	−1
0.088	35	32	−3
0.088	45	43	−2
0.088	49	52	+3
0.085	67	64	−3
0.085	20	22	+2
0.085	33	29	−4
0.085	40	40	0
0.082	80	78	−2
0.082	55	53	−2
0.082	57	58	+1
0.082	61	58	−3

Whole defibrinated blood of an ox was introduced into an isosmotic solution of glycerol, in the proportion of 25 cu. mm. of blood to 25 cc. of 0.3M glycerol. The solution was gently stirred and kept at a temperature of 20° C. and the rate at which hemolysis proceeded determined by the method of Jacobs (1930). Until about 35 minutes after setting up such a system, no hemolysis was found to occur; between 35 and 55 minutes the process proceeded fairly rapidly from 0 to 100 per cent. It was then a simple matter in subsequent experiments to stop the hemolysis at any desired point between these two time intervals by the addition to 25 cc. of the suspension undergoing hemolysis of 25 cc. of a solution at pH 7.4 containing 0.308M NaCl + Na_2HPO_4 and 0.3M glycerol. After the addition of this solution the whole was equilibrated at 20° C. with stirring for a period of one hour, at which time cell counts and estimations of the hemoglobin liberated were made in the manner described in the previous section.

Blood samples from five animals were tested in this manner and the results are summarized in Fig. 3. In this figure, as in the two previous ones, the diagonal line represents the theoretical result that should obtain if the process is all-or-none. It will be observed that in this case the experimental points in these determinations scatter on one side of this line in the direction of an hemolytic process exhibiting a retention of hemoglobin. The deviations, which amount to 5 to 12 per cent, are too great to be accounted for by experimental errors alone.

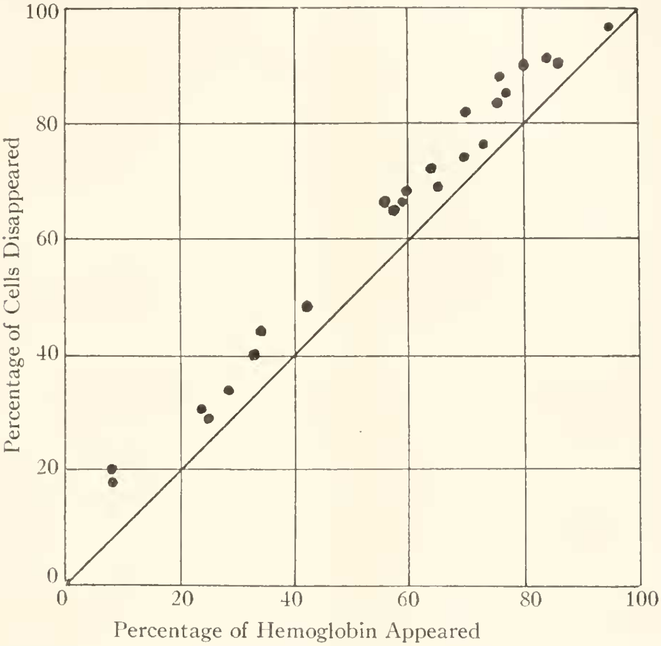

Fig. 3. The liberation of hemoglobin from ox erythrocytes during the early stages of hemolysis by glycerol.

Two possible explanations of these results suggest themselves. The first is that the penetration of the glycerol might so alter the refractive index of a number of the more swollen cells that they do not appear in the count though they still retain all or a part of their hemoglobin. The second is that the hemolytic system may not have attained its final equilibrium at the time the observations were made.

As a test of the former possibility the following experiment was performed. Whole blood of the ox was pipetted into a hypotonic saline solution that would induce a slight degree of hemolysis, in the proportion of 25 cu. mm. of blood in 50 cc. of salt solution at pH 7.4. These solutions were equilibrated for one hour with gentle stirring, at 20° C.

At the end of the equilibration period 25 cc. was removed for cell count and hemoglobin estimation. To the remaining portion was added an equal volume of a solution containing the same concentration of salt plus 0.6M glycerol. Thus the salt concentration remained unchanged, while the solution became isosmotic with respect to glycerol. This latter solution was equilibrated for one hour in the same way, at which time cell counts and hemoglobin determinations were again performed. Due allowance was made for the one-half dilution necessitated by the procedure. The results of several such experiments are presented in Table V.

Table V

Effect of Glycerol on the Refractive Index of the Erythrocyte

Before the Addition of Glycerol			After the Addition of Glycerol		
A. Cells Disappeared	B. Hemoglobin Appeared	B−A	A. Cells Disappeared	B. Hemoglobin Appeared	B−A
per cent	*per cent*		*per cent*	*per cent*	
35	32	−3	40	38	−2
31	29	−2	32	33	+1
28	29	+1	33	32	−1
50	51	+1	52	51	−1

Had the glycerol in any way affected the refractive index of the corpuscles, then the data obtained after the addition of glycerol should have departed from the all-or-none relationship by an amount comparable to that observed in Fig. 3. As no such shift was observed, it may be concluded that glycerol does not appreciably alter the refractive index of the erythrocytes. The explanation, therefore, of the discrepancy between the hemoglobin liberated and the cells disappeared in a hemolytic system of the glycerol type would appear to lie along the lines of the second suggestion, namely the time required for the attainment of equilibrium.

To test this point, whole blood of the ox was equilibrated for one and for four hours, respectively, in hypotonic saline solutions containing varying concentrations of glycerol between 0.03M and 0.3M. As before, 25 cu. mm. of blood was added to 50 cc. of each solution. To make the experimental conditions comparable, half of the solution was removed for the determinations at the end of one hour, the rest being equilibrated in the customary way for an additional period of three hours. The concentrations of salt and of glycerol used are shown in Table VI.

Examination of this table shows that in the absence of glycerol, as in previous experiments, the hemoglobin liberated at the end of either one or four hours agrees within the experimental error with that in the cells which have disappeared. In the presence of glycerol, however, at the end of one hour there is an apparent retention of hemoglobin similar to that indicated in Fig. 3, whereas this had completely disappeared by the end of four hours. In brief, it would appear that at the final equilibrium the conditions are the same as before, but that the final equilibrium is longer in being attained.

TABLE VI

Production by Glycerol of an All-or-none Type of Hemolysis, at Equilibrium

Solution	After 1 Hour			After 4 Hours		
	A. Percentage of Cells Disappeared	B. Percentage of Hemoglobin Appeared	B–A	A. Percentage of Cells Disappeared	B. Percentage of Hemoglobin Appeared	B–A
0.090 M NaCl + Na_2HPO_4	90	88	−2	89	88	−1
	90	90	0	88	89	+1
Same + 0.03 M glycerol	68	60	−8	88	87	−1
Same + 0.10 M glycerol	66	58	−8	93	94	+1
Same + 0.30 M glycerol	34	28	−6	91	92	+1

As to the cause of this delay in the escape of hemoglobin, several possibilities might be suggested. One of the most plausible is that the retention of hemoglobin during the first part of the hemolytic process may be due to a blocking of the hypothetical " pores " by which it is frequently assumed to leave the swollen cells, either because of the relatively large size of the glycerol molecule or because of its adsorption on the walls of the pores. In either case, the effect of the glycerol would be to decrease the surface area through which hemoglobin may leave the cell. The data in Table VI lend confirmation to this view, for not only does the presence of a very small amount of glycerol (0.03M) markedly slow the rate of disappearance of the cells, but it also causes marked temporary retention of hemoglobin. This is true, in spite of the fact that the solution of 0.03M glycerol and of 0.09M NaCl is osmotically equivalent to a 0.105M solution of NaCl, a concentration which of itself rapidly produced about 15 per cent hemolysis. It may further be determined from this table that the initial distance of the hemolytic system from osmotic equilibrium does not influence the amount of retention. As has already been pointed out, the extent of this retention is outside the limits of experimental error of the methods here employed. It averages about 8 per cent as compared with a maximum experimental error of 4 per cent and, as will be observed in Fig. 3, the deviation is consistently in the same direction.

As contrasted with glycerol, ethylene glycol, a closely related substance of lower molecular weight, fails to produce any observable retention of hemoglobin even during the early stages of the hemolytic process. Its behavior is indicated by the data given in Table VII, which were secured by equilibrating for one hour the usual dilution of ox blood in 0.3M ethylene glycol made up in the salt solutions of the concentration indicated in the table. It will be noted that the presence of the ethylene glycol affects neither the rapid attainment of equilibrium nor the all-or-none character of the hemolysis that the hypotonic solutions alone would have exhibited. Except for the lower molecular weight and molecular volume there is no obvious reason why this substance should differ so markedly from glycerol.

Table VII

Hemolysis by Ethylene Glycol

Concentration of $NaCl + Na_2HPO_4$ in 0.3 M Ethylene Glycol	A. Cells Disappeared	B. Hemoglobin Appeared	B–A
	per cent	*per cent*	
0.085	15	19	+4
0.085	19	20	+1
0.080	45	47	+2
0.080	46	46	0
0.078	60	58	−2
0.078	60	62	+2
0.075	75	76	+1
0.075	76	76	0

V

The results that have been obtained fail entirely to confirm Baron's (1929) contention that osmotic hemolysis is a "partial" process. Hemolysis of this type has constantly been found to be an all-or-none phenomenon both under equilibrium conditions and while in progress, except for the temporary retention of hemoglobin that occurs during the early stages produced by a penetrating substance like glycerol.

Even in this case, however, the end-point finally attained by the system is the same as in that found under the simpler conditions where the entrance of water alone is involved.

It was mentioned previously that Baron's results were secured by the use of 1:1 to 1:6 dilutions of whole blood with water. To make cell counts and hemoglobin estimations he added sufficient hypertonic salt solution to make the dilute hemolytic solution isotonic. After obtaining cell counts he then centrifuged the solution and made the hemoglobin estimation on the supernatant fluid. The futility of such a procedure might, however, have been recognized from the results of Bayliss (1924–25), or of Adair, Barcroft and Bock (1921) and others. These workers have clearly demonstrated that when whole blood is diluted with water in proportions such as those mentioned above, the corpuscles swell and lose hemoglobin only to a degree that corresponds with a diffusion equilibrium between the cells and the external solution. If at this point salt is added to make the solution isotonic, the cells shrink, thus trapping in them sufficient hemoglobin so that they again become visible. Bayliss (1924–25) discussed these conditions as follows: " Suppose the ghosts had a volume two and one-half times the volume of the original corpuscles and contained the same concentration of hemoglobin as the surrounding fluid. Then, if on shrinking to their normal size they become more or less impermeable to hemoglobin, they might contain finally a hemoglobin concentration some two to three times that of the external fluid."

It would appear, therefore, that Baron, when making his cell counts, must have included a number of cells whose visibility had been restored after they had previously lost a portion of their hemoglobin. That the hemoglobin was not more completely lost was obviously due to the small volume of the external solution. Under these experimental conditions it is not surprising that no exact correspondence could be obtained between the number of cells that had disappeared and the amount of hemoglobin in the surrounding medium. As has been mentioned, Saslow (1928–29), in his studies on hypotonic saline hemolysis, using the method of Baron, obtained data which contradicted those of the latter author. The reason for this discrepancy is perhaps furnished by Saslow's statement that before making the cell count he pipetted off and discarded the " stromata." The so-called " stromata " were probably cells which had lost their hemoglobin to equilibrium with the surrounding fluid and by discarding them he made his cell count on cells which had not lost hemoglobin, and naturally the cell count corresponded with the hemoglobin in those cells. If he had determined not only the amount of hemoglobin in the cells undestroyed but also that in the surrounding fluid it is likely that the two amounts would have totalled considerably less than 100 per cent. However, this crucial test was not made.

An attempt has been made, by the use of Saslow's dilution of blood with distilled water, to determine the hemoglobin content of the supernatant fluid after " reversion " of the cells by means of sufficient NaCl to make the solution isotonic followed by centrifuging at 5,000 r.p.m. for one and three hours. A clear supernatant fluid is obtained in this way, but further addition of salt to it causes it to become clouded, and microscopic examination shows this cloudiness to be due to the reappearance of cells. If this latter cloudy supernatant fluid is allowed to stand for some fifteen to thirty minutes it again becomes relatively clear and can be made cloudy again by making it still more hypertonic with salt. A short time later it again becomes clear. Thus, in the method of Saslow, and also in that of Baron, the " clear " supernatant fluid really must contain cells which have approximately the same specific gravity and, more important, the same hemoglobin content as the surrounding fluid. Because of this circumstance hemoglobin determinations on the supernatant liquid of very concentrated suspensions, whether made directly, as by Baron (1928), or indirectly, as by Saslow (1928–29), are unreliable.

Consideration of these facts leads to the conclusion that only when conditions are created such that the volume of the cells at the time of hemolysis is relatively small as compared with that of the surrounding fluid, can the nature of hemolysis be determined. Dilution of whole blood in hypotonic salt solution in the proportion of 1:2,000 adopted in these experiments leads to practically infinite dilution of the hemoglobin content of the hemolyzed cells. Under these conditions " ghost " cells and the phenomenon of " reversion " introduce no complications and it becomes possible to demonstrate that osmotic hemolysis is an all-or-none phenomenon, and that the term " percentage hemolysis " has a real significance.

SUMMARY

1. Hemolysis produced by hypotonic sodium chloride is of an all-or-none type, that is, hemoglobin either fails to escape from the erythrocyte or does so completely up to the point permitted by the attainment of a diffusion equilibrium in the system.

2. The same all-or-none character is observed when, instead of permitting the hemolytic process to proceed to its original equilibrium position, it is stopped at an intermediate point by the addition of sodium chloride.

3. In hemolysis in solutions of penetrating substances such as glycerol and ethylene glycol, the final equilibrium obtained after checking the process by the addition of sodium chloride likewise indicates an all-or-none relationship.

4. In glycerol solutions the liberation of hemoglobin lags somewhat behind the disappearance of the cells and the final equilibrium is rather slowly attained.

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